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Commitment of Annexin A2 in recruitment of microRNAs into extracellular vesicles



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ABSTRACT

Extracellular vesicles (EVs) contain microRNAs (miRNAs). However, the exact molecular mechanisms of the recruitment of miRNAs in EVs are not well characterized. Based on proteomic analysis, we identified that silencing of Annexin A2 (ANXA2) significantly decreased the amount of miRNAs in EVs. In addition, microarray analysis revealed that ANXA2 regulated the loading of miRNAs into EVs in a sequence independent manner. Lastly, immunoprecipitation analysis confirmed that ANXA2 could bind miRNAs in EVs in the presence of Ca²⁺. These observations demonstrate that ANXA2 plays an important role in the packaging process of miRNAs into EVs.

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1. Introduction

Small non-coding RNA microRNAs (miRNAs) regulate gene expression at the post-transcriptional level by binding sequence-specific sites within the 3' untranslated region of target mRNAs [1]. In addition, miRNAs can fine-tune various biological processes, including development, organogenesis, metabolism, and homeostasis [2]. miRNAs have been found in human body fluids despite the abundant presence of ribonucleases (RNases) [3]. This finding has led to the proposal of a scenario in which miRNAs could be packaged in certain RNase-resistant containers when they are secreted out of cells. Indeed, it has been reported that miRNAs are present in extracellular vesicles (EVs), as has been described for various body fluids [4]. Moreover, recent evidence suggests that

EV miRNAs in body fluids can exhibit disease-specific signatures and can be used as biomarkers [5]. Another major interest is the notion that EVs allow the transfer of miRNAs between cells. Remarkably, such transferred miRNAs can be functional once they have been incorporated into the recipient cells [6–8].

The findings have raised the idea that EVs could be used as carriers for effective drug delivery strategies. Indeed, Ohno et al. generated modified EVs derived from HEK293 cells expressing EGF or GE11 peptide, another ligand for the EGF receptor, on their surfaces. These modified EVs successfully delivered let-7a miRNA to EGFR-expressing xenografted breast cancer tissue in immunodeficient mice [9]. However, the precise molecular mechanisms responsible for miRNA loading into EVs remain unclear, preventing EVs from being considered for further development and clinical applications of these vesicles.

Considering that miRNAs generally exist as complexes with proteins, it is conceivable that certain proteins form miRNA–protein complexes and recruit bound miRNAs into EVs. Intriguingly, however, multiple studies reported that the Argonaute2 protein, which binds to RNAs including miRNAs and plays a central role in RNAi, does not bind to miRNAs in EVs [10,11]. Thus, other RNA-binding proteins appear to contribute to miRNA–protein complex formation and the subsequent loading of miRNAs into EVs. Of note, it is likely that RNA-binding proteins typically bind

Abbreviations: miRNA, microRNA; EV, extracellular vesicle; qRT-PCR, quantitative RT-PCR; siRNA, small interference RNA; RNase, ribonuclease; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ANXA1, Annexin A1; ANXA2, Annexin A2; ANXA5, Annexin A5; ANXA6, Annexin A6; EEF1G, elongation factor 1-γ; EEF2, elongation factor 2; HIST4H4, histone H4

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to miRNAs non-selectively because most RNA-binding proteins recognize the secondary structure of miRNAs, but not their specific sequence of them [12].

Based on this background, in this study, we explored proteins that commonly recruit miRNAs into EVs. Following our proteomic analysis, we identified 8 candidate proteins and focused on the calcium-dependent binding protein Annexin A2 (ANXA2) as the most promising candidate for further analyses. Indeed, a miRNA microarray analysis showed that both control and ANXA2 knock-down cell-derived EVs contain similar miRNA profiles, despite a decrease in the quantity of loaded miRNAs in the ANXA2-knockdown EVs, suggesting ANXA2-mediated non-selective loading of miRNAs into EVs. Knockdown and overexpression of ANXA2 decreased and increased, respectively, the total amounts of miRNAs in EVs without changing the intracellular levels of the miRNAs. Taken together, we propose that the loading process of miRNAs into EVs is mediated by ANXA2 in a sequence independent manner.

2. Materials and methods

2.1. Reagents

Mouse monoclonal anti-CD9 (sc-59140), mouse monoclonal anti-human CD81 (sc-23962) and goat polyclonal anti-Alix (sc-49268) were purchased from Santa Cruz Biotechnology. Mouse monoclonal anti-ANXA2 (610068) was obtained from BD Biosciences. The recombinant human ANXA2 protein was purchased from ProSpec. Anti-FLAG M2 magnetic beads were obtained from Sigma–Aldrich.

2.2. Plasmids

The pcDNA3 (V790-20) was purchased from Addgene. For the immunoprecipitation assay, a FLAG-tagged ANXA2 overexpression vector was constructed by introducing the following sequence at the BamHI and XbaI sites in the multi-cloning sequence of the pcDNA3 vector: 5'-CGGGATCCGCCGCCATGGACTACAAGGACG ATGATGACAAGGGCTCTACTGTTACGAAATC-3' (sense) and 5'-GCTCTAGATTAGTCATCTCCACCACACAGGTAC-3' (antisense); the FLAG sequence is underlined.

2.3. Cell culture

The human breast cancer cell line MDA-MB-231-luc-D3H2LN (Xenogen), the human prostate cancer cell line 22Rv1 (ATCC), and the human prostate cancer cell line PC3 (ATCC) were cultured in RPMI 1640 (Invitrogen) containing 10% heat-inactivated fetal bovine serum and an antibiotic solution at 37 °C in 5% CO₂. The human osteosarcoma cell line 143B (ATCC) and the human hepatocellular carcinoma cell line Huh-7 (RIKEN BRC) were cultured in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% heat-inactivated fetal bovine serum and an antibiotic solution at 37 °C in 5% CO₂.

2.4. Preparation of conditioned medium and EVs

Cells were seeded in 15 cm dish at 3×10^6 cells (20 mL per dish). Before collection of the culture medium, the cells were washed with PBS, and the medium was switched to Advanced RPMI containing an antibiotic–antimycotic and 2 mM L-glutamine (without FBS). The conditioned medium was collected after incubation for 48 h. EVs were purified by differential centrifugation as described previously [8]. To thoroughly remove cellular debris, the supernatant was filtered with a 0.22- μ m filter unit (Millipore). Then, the CM was ultracentrifuged at 110000 \times g for 70 min at 4 °C.

The pellets were washed with 11 mL PBS, and after ultracentrifugation, they were resuspended in PBS. The putative EV fraction was measured with regard to protein content using a Quant-iT™ Protein Assay with a Qubit® 2.0 Fluorometer (Invitrogen). The amount of EVs obtained is $\sim 2 \mu$ g from 10^6 cells in PC3 cells. For proteomic analysis, we collected EVs from 500 mL (7.5×10^7 cells) of conditioned medium. For qRT-PCR, we collected EVs from 40 mL (6×10^6 cells) of conditioned medium.

2.5. Preparation of EV lysates

Whole EV lysates were prepared using Mammalian Protein Extract Reagent (Thermo Scientific). Then, whole EV lysates were transferred to a 1.5 mL tube and treated by sonication.

2.6. Proteomic analysis

For the proteomic analysis, we used EVs purified from 500 mL of conditioned medium for each cell line. A mass spectrometric analysis was carried out using LC–MS/MS [13,14]. The LC–MS/MS parameter and methods were described previously [15]. The database search was performed with MASCOT Deamon (Matrix Science) [16–18]. The generated pkl files were submitted to SWISS-PROT (12012_02) and NCBI nr (20120303); the search parameters were as follows: fixed modifications, carbamidomethyl; variable modifications, oxidation (M); missed cleavages, up to 1; monoisotopic peptide tolerance, 1.0 Da; taxonomy, human and MS/MS tolerance, 0.5 Da. To automatically eliminate very-low-scoring random peptide matches, the ion score cutoff was set to 20; thus homologous proteins are more likely to collapse into a single hit, which avoids the need to choose between them. MOWSE scores are reported as $-10 \times \log_{10}(P)$, where P is the absolute probability; a probability of 10–20 thus became a score of 200 [19].

2.7. Transient transfection assays

Transfection of a small interfering RNA (siRNA) was accomplished using the DharmaFECT transfection reagent (Thermo Scientific) according to the manufacturer's protocol. AllStars Negative Control siRNA (Qiagen) was used as a negative control.

2.8. Measurement of size distribution by nanoparticle tracking analysis

Nanoparticle tracking analysis was conducted using the NanoSight LM10-HS system (NanoSight Ltd.) for EVs that were resuspended in PBS. We quantified the relative number of released EVs collected from the distinct culture conditions concomitantly for each experiment to directly compare the treated and control samples.

2.9. Cell proliferation assay (MTS assay)

A total of 5000 cells per well were seeded in 96-well plates; the following day, the cells were transfected with siRNAs. After 3 days of culture, cell viability was measured using Cell Counting Kit-8 (Dojindo) according to manufacturer's protocol. The absorbance was measured at 450 nm using the EnVision system (Wallac).

2.10. Isolation of miRNAs and quantitative real-time PCR (qRT-PCR)

Total RNAs were extracted from cultured cells using QIAzol and miRNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. PCR was performed in 96-well plates using a 7300 Real-Time PCR System (Applied Biosystems). TaqMan qRT-PCR kits were purchased from Applied Biosystems. Reverse transcription (Applied

Biosystems) and TaqMan quantitative PCR (Applied Biosystems) were performed according to manufacturer's instructions.

2.11. Immunoblot analysis

SDS–PAGE gels were calibrated using Precision Plus protein standards (161-0375) (Bio-Rad), and anti-ANXA2 (1:200), anti-CD9 (1:200), anti-CD81 (1:200) and anti-Alix (1:200) were used as primary antibodies. A peroxidase-labeled anti-mouse secondary antibody was used at a dilution of 1:1000. The primary-antibody-recognized protein bands were visualized using a peroxidase-conjugated secondary antibody and enhanced chemiluminescence with an ECL Plus Western blotting detection system (RPN2132; GE HealthCare). Then, Luminescent images were captured using a Luminolmager (LAS-3000; FujiFilm Inc.).

2.12. Immunoprecipitation–qRT-PCR

Anti-FLAG M2 magnetic beads (20 μ L) (Sigma–Aldrich) were washed 3 times in TBS containing 1 mM Ca^{2+} , resuspended in 1 mL of EV sample, and incubated overnight at 4 °C with gentle mixing. The beads were then washed 3 times in TBS containing 1 mM Ca^{2+} to remove all of the non-specifically bound proteins.

For the qRT-PCR analysis, 500 μ L of QIAzol (Qiagen) was added, and the samples were vortexed prior to use.

2.13. Statistical analysis

Data are presented as the mean \pm SD, and the experimental points show the average of at least triplicates. All experiments were repeated at least 3 times. Statistical analyses were performed using Student's *t*-test.

3. Results

3.1. EV proteins identified by proteomic analysis

To identify the molecules responsible for the common loading of miRNAs into EVs, we performed a proteomic analysis using LC–MS/MS (Table S1). For this proteomic analysis, EVs were isolated by ultracentrifugation from 4 human cancer cell lines, including breast cancer, prostate cancer, osteosarcoma, and hepatocellular carcinoma lines. From the proteomic data obtained, we selected 8 candidates—glyceraldehyde 3-phosphate dehydrogenase (GAPDH), annexin A1 (ANXA1), ANXA2, annexin A5 (ANXA5), annexin A6 (ANXA6), elongation factor 1- γ (EEF1G),

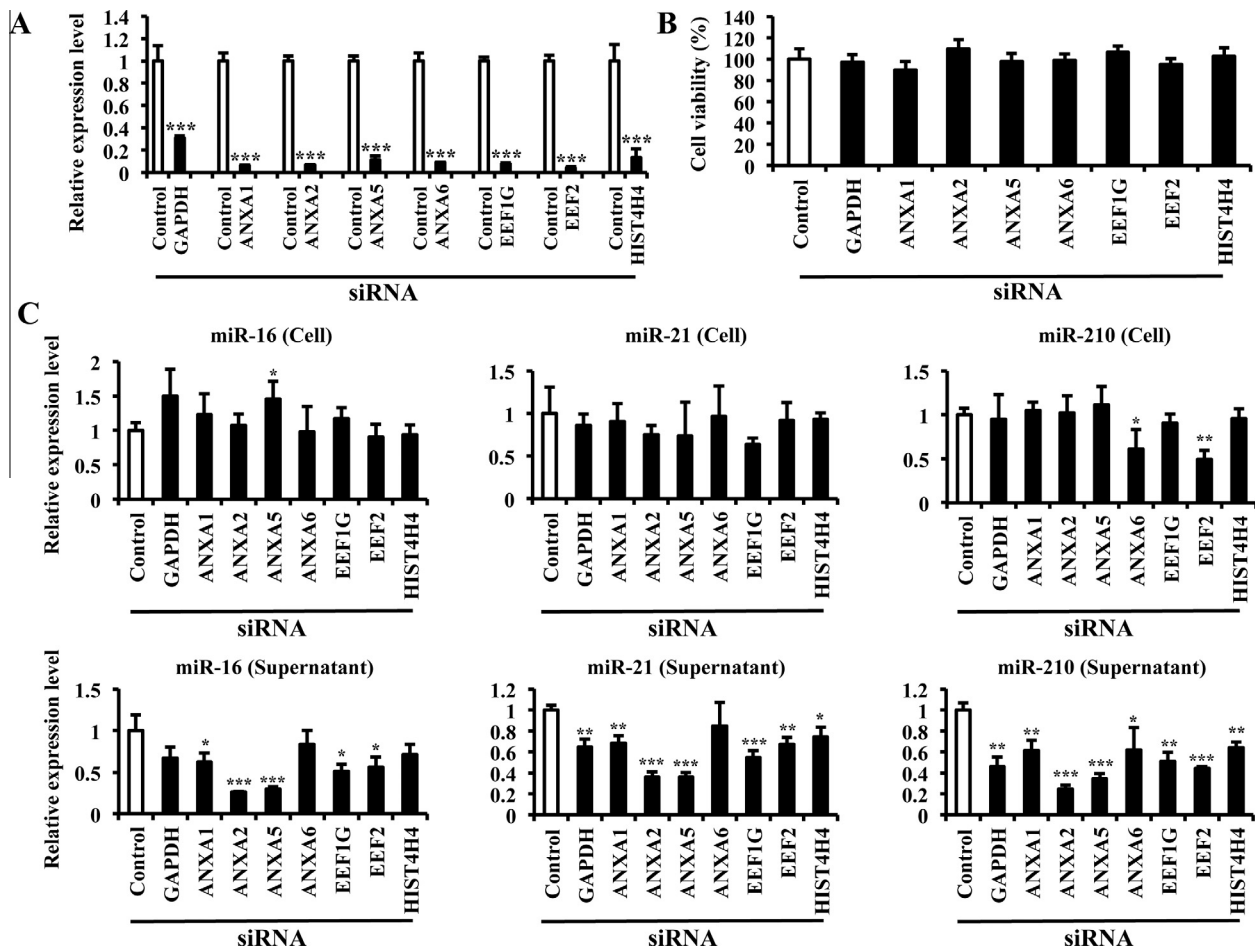


Fig. 1. ANXA2 is involved in the extracellular export of miRNAs. PC3 cells were transfected with control, GAPDH, ANXA1, ANXA2, ANXA5, ANXA6, EEF1G, EEF2, and HIST4H4 siRNA. (A) After 2 days of culture, cell extracts were subjected to qRT-PCR. The expression level of the cells treated with the control siRNA was defined as 1.0. (B) After 3 days of culture, cell growth was assessed by an MTS assay. The values on the y-axis are relative to the cell viability of the control siRNA treatment, which is defined as 100. (C) The cell lysate (upper panels) and the conditioned medium (lower panels) were subjected to qRT-PCR. Expression levels of miR-16 (left panels), miR-21 (middle panels), and miR-210 (right panels) were analyzed. All data are shown as the mean \pm SD. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ and the experimental points show the average of at least triplicates. All experiments were repeated at least 3 times. Statistical analyses were performed using Student's *t*-test.

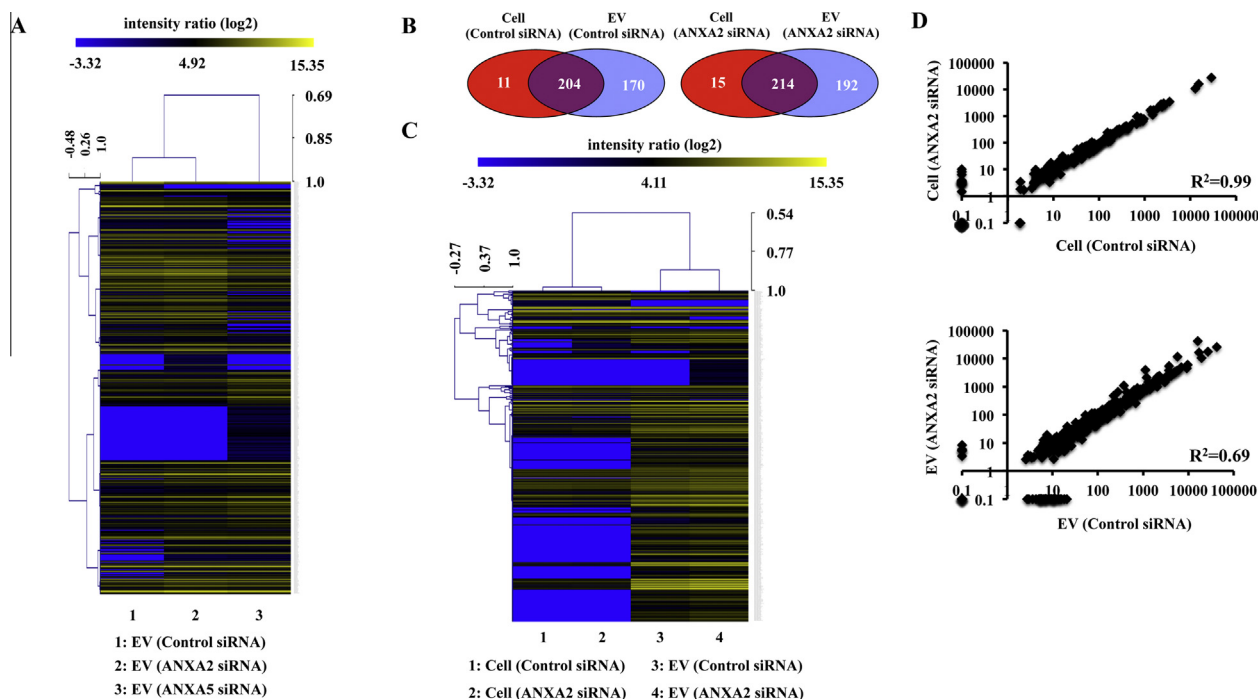


Fig. 2. ANXA2 recruits miRNAs into EVs. EVs were collected from PC3 cells transiently transfected with control, ANXA2, and ANXA5 siRNA. Both cells and EVs were subjected to miRNA microarray analysis. (A) The clustering analysis of EVs is shown. (B) Number of miRNAs in EVs and cells detected by the microarray analysis. (C) The clustering analysis of EVs and cells is shown. Yellow to Blue, color-range gradient displaying the expression level. (D) Scatter plots of correlation coefficient analysis were performed.

elongation factor 2 (EEF2), and histone H4 (HIST4H4)—that were commonly present in EVs from at least 2 cancer cell lines (Fig. S1).

3.2. Inhibition of ANXA2 expression decreases the amount of extracellular miRNAs

To examine whether these 8 candidate proteins can participate in the loading process of miRNAs into EVs, we investigated both intracellular and extracellular miRNA levels by qRT-PCR after knocking down each of these genes. We analyzed the expression of miR-16, miR-21, and miR-210, as these 3 miRNAs have been shown to be abundant in EVs [20]. Following transfection with siRNAs, we confirmed that each candidate gene was successfully silenced (Fig. 1A) without any cytotoxic effects (Fig. 1B). Notably, after the silencing of ANXA1, ANXA2, ANXA5, and EEF1G, the amount of all the tested miRNAs significantly decreased in the EVs, though the corresponding intracellular expression levels of these miRNAs were not changed (Fig. 1C). Among these proteins, ANXA2 and ANXA5 silencing showed the most significant results. Consequently, we hypothesized that ANXA2 and ANXA5 could represent major players in the recruitment of miRNAs into EVs and decided to focus on these proteins for further investigation.

3.3. ANXA2 participates in non-selective loading of miRNAs into EVs

To reveal whether ANXA2 and ANXA5 globally recruit miRNAs without sequence selectivity, we performed a microarray analysis on EVs derived from ANXA2-knockdown, ANXA5-knockdown, and control cells and found that ANXA5 silencing affected EV miRNA profile patterns more significantly than ANXA2 silencing (Fig. 2A). These results demonstrated that ANXA2 participates in the loading of miRNAs into EVs in a relatively non-selective way. Thus, we focused on ANXA2 for further investigation and analyzed microarray data for EVs derived from ANXA2-knockdown and control cells, since as noted in the Introduction, our primary goal is to

exploration for proteins that commonly recruit miRNAs into EVs (Table S2). This analysis revealed that 214 miRNAs could be found in both cells and EVs after ANXA2 siRNA transfection (Fig. 2B and Table S2). Interestingly, it appeared that ANXA2 silencing did not affect the miRNA profiles observed in cells and EVs (Fig. 2C). Next, a cellular miRNA comparison between ANXA2 silencing and the control showed a high degree of concordance ($R^2=0.99$) (Fig. 2D). In addition, the comparison between ANXA2 silencing and the control in EVs showed good concordance ($R^2=0.69$) (Fig. 2D).

3.4. Characterization of ANXA2 in EVs

To confirm that ANXA2 is present in EVs, we analyzed the amount of ANXA2 in EVs using western blotting. As shown in Fig. 3A, the ANXA2 protein was evident in both EVs and cells. In addition, the levels of ANXA2 in EVs dramatically decreased after ANXA2 silencing (Fig. 3B), indicating that the abundance of ANXA2 in EVs is correlated to the expression level of ANXA2 in cells. To eliminate the possibility that ANXA2 silencing could decrease the number of secreted EVs, the particle number and size of EVs were measured by nanoparticle tracking analysis. The number of EVs derived from PC3 cells after ANXA2 silencing was not changed compared to control siRNA-treated cells (Fig. 3C), with the size of EVs derived from PC3 cells showing a peak between 100 and 150 nm, regardless of the level of ANXA2 (Fig. 3D). This finding indicates that ANXA2 did not affect the EV biogenesis and secretion process. To explore the relationship between ANXA2 and EV miRNA abundance, we isolated EVs from 2 prostate cancer cell lines expressing high (PC3) or low (22Rv1) levels of ANXA2 (Fig. 3E). A positive correlation between ANXA2 and miRNA levels in EVs was observed, whereas the intracellular expression levels of these miRNAs did not show any correlation with ANXA2 between the PC3 and 22Rv1 cells (Fig. 3F). These results strongly suggest that ANXA2 may participate in the loading process of miRNAs into EVs.

3.5. ANXA2 recruits a set of miRNAs into EVs

To clarify whether ANXA2 can regulate the recruitment of miRNAs into EVs, we examined a specific set of miRNAs, including miR-16, miR-21, miR-24, miR-29a, miR-100, miR-125, let-7a, and let-7b, as our previous study indicated the abundance of these miRNAs in PC3 cell-derived EVs [20]. All of the tested miRNAs in the EVs derived from ANXA2-silenced cells were found to be downregulated (Fig. 4A). Next, the amount of EV miRNA was analyzed in ANXA2-overexpressing cells. Following transfection with an ANXA2 overexpressing vector, increased levels of ANXA2 in both the cells and EVs were confirmed by qRT-PCR and western blotting (Fig. 4B and C). The results showed that ANXA2 overexpression increased the level of miR-16 in EVs without affecting the cellular level of miR-16 (Fig. 4D). These results reinforced our evidence that ANXA2 regulates the packaging process of miRNAs into EVs.

3.6. ANXA2 is associated with miRNAs in EVs

To examine the binding ability of ANXA2 with miRNAs, we designed a FLAG-ANXA2 overexpression vector for immunoprecipitation (Fig. 5A). After expression of FLAG-ANXA2 in HEK293 cells,

ANXA2 was immunoprecipitated from the EV lysate using an anti-FLAG antibody (Fig. 5B), revealing that the ANXA2 fraction contained a higher amount of miRNAs in the presence of Ca^{2+} at 1 mM (Fig. 5C). These findings collectively indicated that ANXA2 could specifically bind miRNAs in EVs in a calcium-dependent manner.

4. Discussion

Although a number of reports have suggested that miRNAs in EVs have the potential to be used in RNAi-based therapy and for diagnosis of various diseases, the loading process of miRNAs into EVs remains unclear. In this study, we demonstrated that ANXA2 plays a major role in the loading of miRNAs into EVs.

As noted in the Introduction section, the goal of this study was to search for proteins that commonly recruit miRNAs into EVs, and we identified ANXA2 as such a protein. The EV database ExoCarta (<http://exocarta.org>) provides evidence that over 4000 proteins can be contained within EVs, including a calcium channel-associated protein, CACNA2D4, and an mRNA-binding protein, SERBP1 [21]. However, we cannot rule out the possibility that other proteins might contribute to miRNA loading into EVs. Indeed,

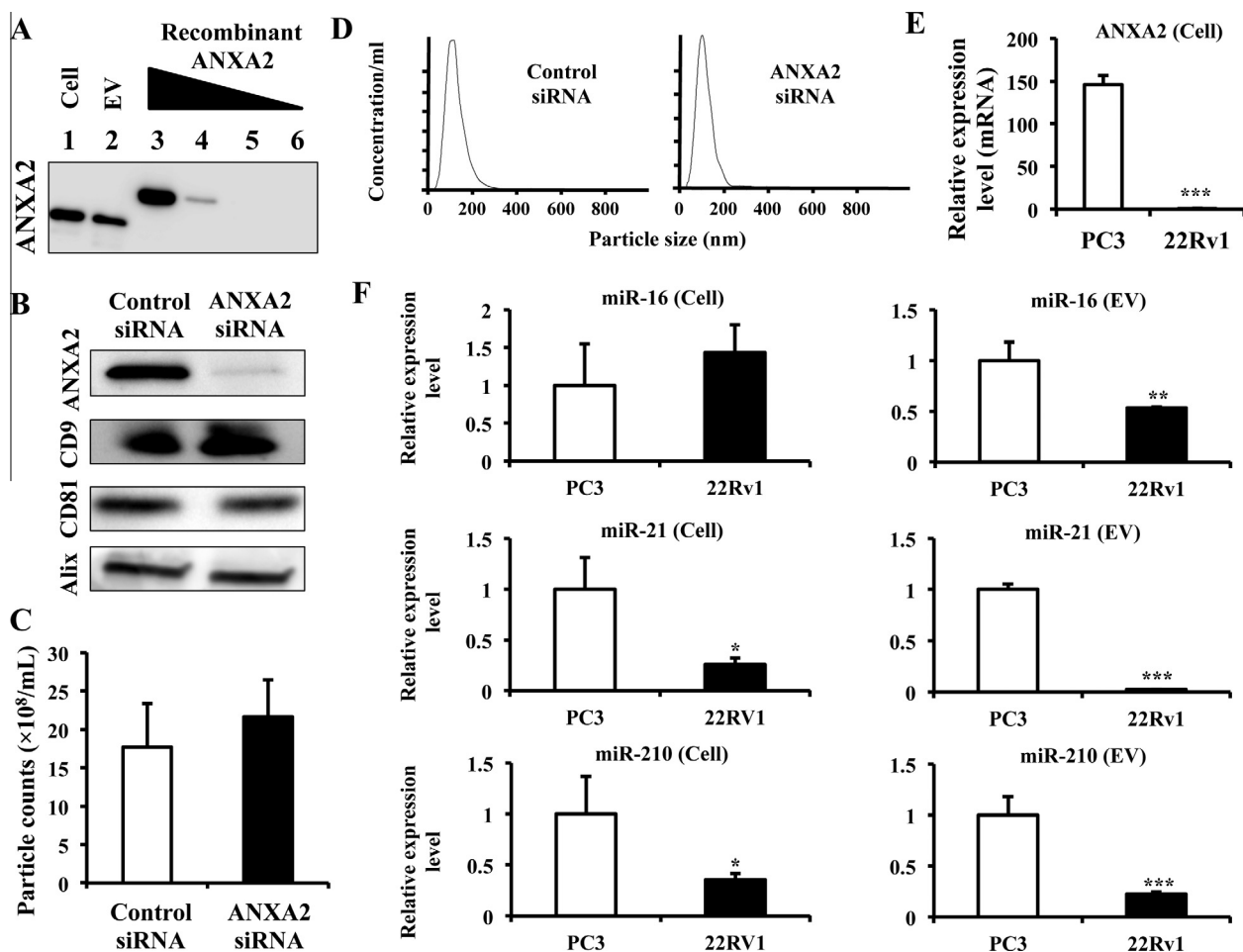


Fig. 3. ANXA2 is identified in EVs. (A) Whole-cell lysates and EVs were collected from PC3 cells. ANXA2 expression was analyzed using immunoblotting. Lane 1: cell lysate (500 ng), lane 2: EV (500 ng), lane 3: recombinant ANXA2 (10 ng), lane 4: recombinant ANXA2 (1 ng), lane 5: recombinant ANXA2 (0.1 ng), and lane 6: recombinant ANXA2 (0.01 ng). EVs were collected from PC3 cells transiently transfected with control or ANXA2 siRNA. (B) A 500 ng sample of EV protein was used for the detection of ANXA2, CD9, CD81, or Alix. (C) Nanoparticle concentrations per mL of EVs. (D) Size distribution of EVs determined by the NanoSight system. Data represent the average size distribution profile of the average size for each sample ($n = 3$). (E) ANXA2 expression was examined by qRT-PCR in PC3 cells and 22Rv1 cells. (F) Whole-cell lysates (left panels) and EVs (right panels) were collected from PC3 cells and 22Rv1 cells. Expression levels of miR-16 (upper panels), miR-21 (middle panels), and miR-210 (lower panels) were analyzed using qRT-PCR. All data are shown as the mean \pm SD. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ and the experimental points show the average of at least triplicates. All experiments were repeated at least 3 times. Statistical analyses were performed using Student's *t*-test.

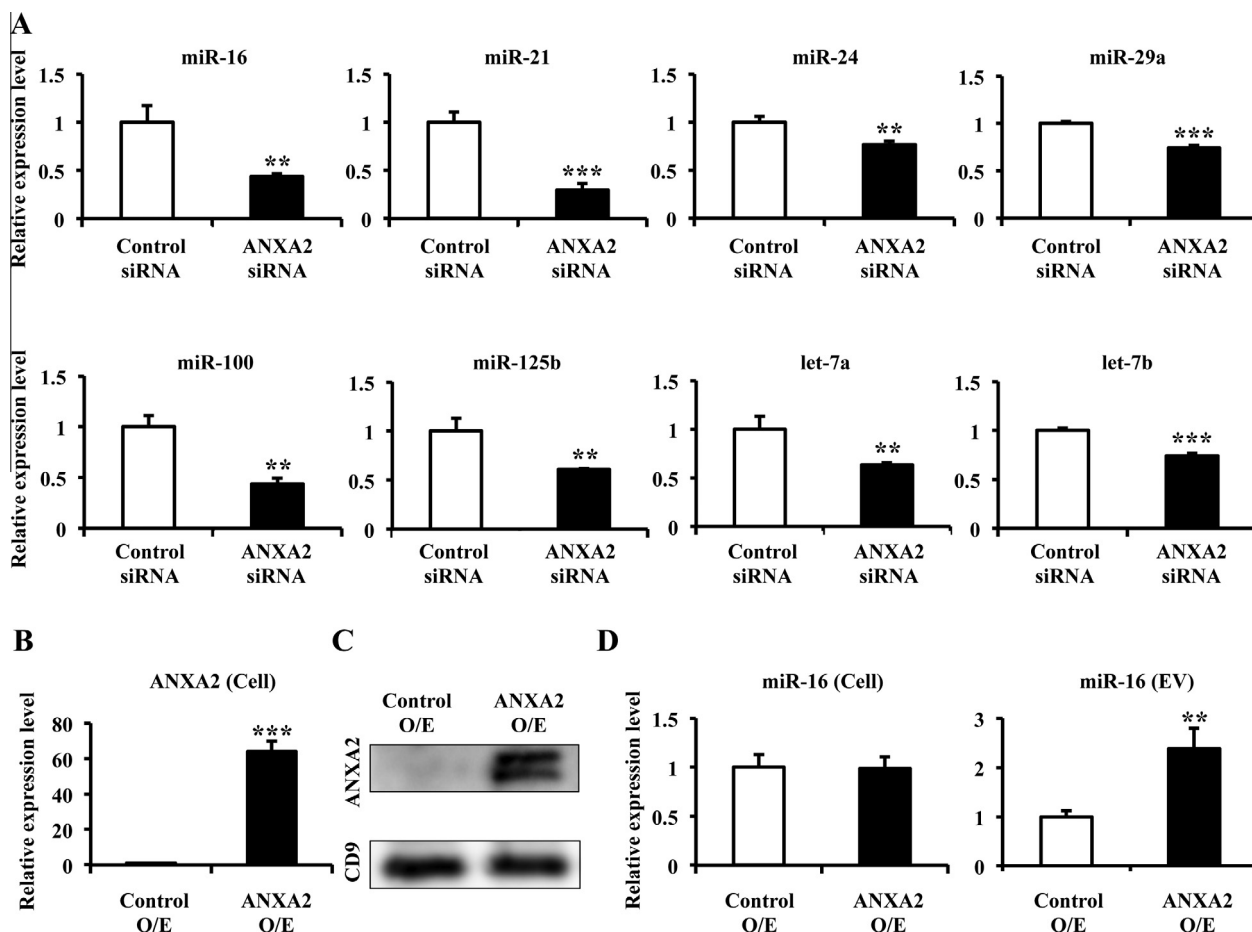


Fig. 4. ANXA2 contributes to the process of miRNA loading into EVs. (A) EVs were collected from PC3 cells transiently transfected with control or ANXA2 siRNA and subjected to miRNA qRT-PCR. (B) HEK cells were transiently transfected with a control or ANXA2-overexpressing vector. After 2 days of culture, cell and EV extracts were subjected to qRT-PCR. (C) A 100 ng sample of EV proteins was used for the detection of ANXA2 (upper panel) or CD9 (lower panel). (D) Expression levels of miR-16 were analyzed in cells and EVs. All data are shown as the mean \pm SD. * P < 0.05, ** P < 0.01, and *** P < 0.001 and the experimental points show the average of at least triplicates. All experiments were repeated at least 3 times. Statistical analyses were performed using Student's *t*-test.

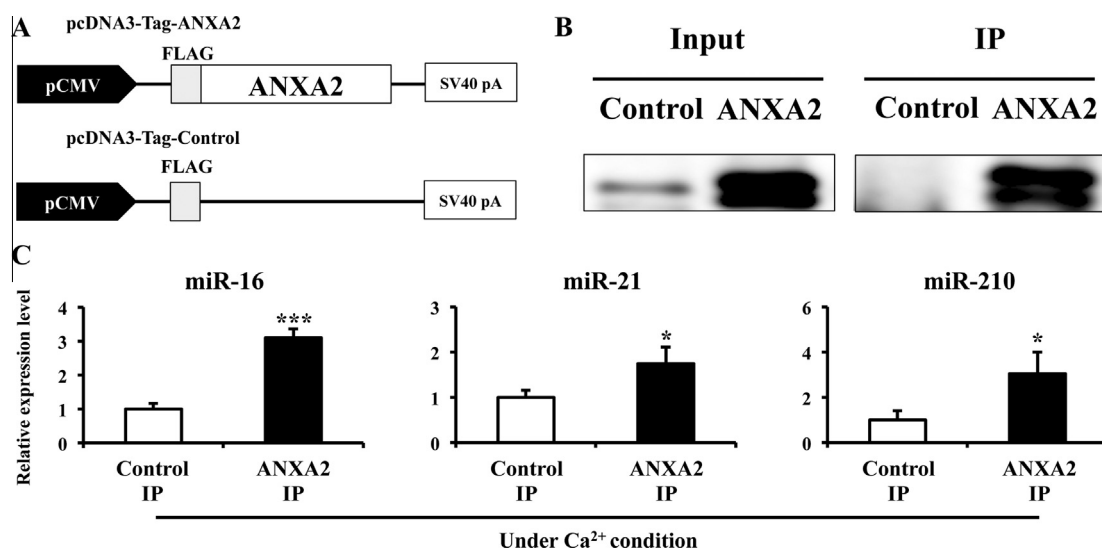


Fig. 5. ANXA2 is associated with miRNAs in EVs. (A) Schematic representation of the FLAG-tagged ANXA2-overexpressing vector. The coding sequence of ANXA2 was inserted into pcDNA3 and fused with the FLAG tag at the N-terminus. SV40A indicates an SV40 polyadenylation signal. HEK293 cells were transiently transfected with the control or FLAG-tagged ANXA2-overexpressing vector. (B) ANXA2 was immunoprecipitated from EV lysate and immunoblotted using an anti-ANXA2 antibody. (C) Expression levels of miR-16 (left panels), miR-21 (middle panels), and miR-210 (right panels) in ANXA2 immunoprecipitates from EV lysates with Ca^{2+} were analyzed. All data are shown as the mean \pm SD. * P < 0.05, ** P < 0.01, and *** P < 0.001 and the experimental points show the average of at least triplicates. All experiments were repeated at least 3 times. Statistical analyses were performed using Student's *t*-test.

previous studies have reported that sumoylated hnRNPA2B1 sorts specific miRNAs into EVs [22]. In addition, we found that the knockdown of ANXA1, ANXA5, and EEF1G could also decrease the abundance of miRNAs in culture supernatant (Fig. 1C). Furthermore, we confirmed by qRT-PCR that ANXA5 silencing decreased the amount of miR-16 and miR-21 in EVs (data not shown). Although ANXA2 regulated the loading of miRNAs into EVs in a sequence independent manner, our miRNA microarray analysis showed that ANXA2 silencing decreased the relative abundance of only 6 miRNAs (Fig. S2). These observations suggest that ANXA2 might have a selective affinity for a small number of specific miRNAs during their packaging into EVs. Notably, we demonstrated that ANXA5 silencing more significantly affected EV miRNA profiles compared to ANXA2 silencing (Fig. 2A). These observations reinforce our thesis that the role of ANXA2 in the loading process of miRNAs into EVs is largely non-selective.

Interestingly, we found that the ANXA2-associated enrichment of miRNAs in EVs was Ca^{2+} dependent. Accordingly, some reports have supported evidence that EVs contain a high concentration of Ca^{2+} . For instance, secretory granules contain high amounts of Ca^{2+} ions in endocrine cells [23]. Emmanouilidou et al. also reported that an increase in the intracellular Ca^{2+} concentration can stimulate EV secretion [24]. Moreover, monensin, which activates Ca^{2+} entry by reversing the activity of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger, induces the accumulation of Ca^{2+} in the enlarged multivesicular bodies, from which EVs are derived [25]. Thus, our results now propose a hypothesis that the enrichment of Ca^{2+} in EVs could promote in the loading process of miRNA mediated by ANXA2.

We found that ANXA2 binds miRNAs in EVs (Fig. 5C). However, we cannot exclude the possibility that ANXA2 interacts indirectly with miRNAs. Indeed, it has been reported that ANXA2 has the ability to be associated with several proteins such as p11 and tPA [26,27]. Moreover, ANXA2 can bind directly to both mRNA and ribonucleotide homopolymers [28,29]. Thus, there is a possibility that ANXA2 recruits miRNAs via its interaction with other miRNA-associated proteins. We are planning to investigate this possibility in our future studies.

The discovery of EVs as carriers of miRNAs led to the proposal that EVs could be used as exogenous miRNA delivery systems for gene therapy [30]. Indeed, our group previously reported that EVs from normal epithelial prostate cells inhibit the proliferation of prostate cancer cells in vitro; in this study, the tumor-suppressive miRNA miR-143 was able to inhibit cell growth through the downregulation of KRAS and ERK5 expression after its transfer into recipient cells [20]. The main limitation of EV-based therapeutic strategies is the requirement of large amounts of EVs. Therefore, the development of effective methods for significantly increasing the amount of miRNAs in EVs remains a major challenge. In the present study, we found that ANXA2 overexpression increased the amount of EV miRNAs (Fig. 4D). Thus, overexpressing ANXA2 could represent a promising strategy to significantly increase the amount of miRNAs packaged into EVs.

Our findings indicate that ANXA2 plays a crucial role in the loading process of miRNAs into EVs. To the best of our knowledge, our results present the first evidence of a molecular process underlying miRNA loading into EVs in a sequence independent manner. However, further studies will be still required for the entire understanding of the mechanisms governing the miRNA loading into EVs. For example, it is not clear whether ANXA2 binds directly to miRNAs, or requires any other mediating molecules such as Ago2. Although Ago2 is one of the most important miRNA-binding proteins, a previous study reported that EVs do not contain Ago2 [31]. Instead, it is suggested that Ago2 complexes carry a population of circulating microRNAs that are not associated with EVs in human plasma [32]. Thus, further investigation will be

required to clarify (1) whether ANXA2 can bind directly to its target miRNAs, and if it is not the case, (2) what molecule mediates the ability of ANXA2 to form complexes with miRNAs.

In conclusion, our study provides evidence for an ANXA2-dependent loading mechanism of miRNA into EVs. To our knowledge, this is the first report that highlights such a specific protein in the recruitment of miRNAs into EVs. Importantly, we reveal that ANXA2 regulates the loading of miRNA into EVs in a sequence independent manner. As ANXA2 overexpression significantly increased the amount of EV miRNAs, we anticipate that modulating ANXA2 may represent an interesting strategy to enrich EV miRNAs for future biomedical applications.

Conflict of interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.febslet.2015.11.036>.

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